

# Coinfection with “*Rickettsia sibirica* subsp. *mongolotimonae*” and *Rickettsia conorii* in a Human Patient: a Challenge for Molecular Diagnosis Tools

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**Rickettsioses are zoonoses transmitted by vectors. More than one agent can coexist in vectors. Although vectors may transmit more than one microorganism to humans, information on dual infections is scarce. We present a case of a patient with an atypical rickettsiosis diagnosis in whom two species of *Rickettsia* were detected.**

## CASE REPORT

A 46-year-old woman was admitted to Bellvitge University Hospital in May with fever (38°C) as well as a retroauricular adenopathy and a subsequent preauricular and lateral cervical adenopathy that had lasted for 10 days. On examination at admission, she was afebrile and she had a necrotic eschar on the scalp (tache noire), face swelling, and a rash on her upper extremities. Laboratory examination showed normal values, except for the presence of activated lymphocytes and increased levels of alanine aminotransferase/aspartate aminotransferase, lactate dehydrogenase, and gamma-glutamyl transpeptidase. Testing of the patient's serum for toxoplasma and cytomegalovirus were negative. She reported that she had a dog and she had observed ticks on it recently.

Tick-borne lymphadenopathy (TIBOLA) (recently called scalp eschar and neck lymphadenopathy syndrome [SENLAT]) caused by *Rickettsia slovaca* was suspected. As a consequence, a regimen of doxycycline (100 mg every 12 h for 4 days) was prescribed. The patient's condition improved. She was discharged 5 days after admission. The patient underwent periodical clinical evaluations for 49 days until her symptoms resolved completely.

At admission, an acute-phase serum sample, a blood sample, and a scalp eschar biopsy specimen were collected. Serum was evaluated by indirect immunofluorescence assay (IFA). Antibodies against *Rickettsia conorii* were tested for using a commercial antigen (*R. conorii* spot; bioMérieux, Marcy l'Étoile, France). Antibodies against *R. slovaca* were tested for using an *R. slovaca* antigen kindly provided by the Unité de Rickettsies, Marseille, France. IgG antibodies against *R. conorii* antigen (titer > 5,120) were detected, whereas the IFA using *R. slovaca* antigen was negative. As neither clinical nor epidemiological variables were typical of Mediterranean spotted fever (MSF, whose etiological agent is *R. conorii*), and IFA for *R. slovaca* was not positive, a more accurate diagnosis was carried out.

DNA was obtained from the whole-blood sample, the scalp eschar biopsy specimen, and the liquid that contained the scalp eschar biopsy specimen. For this purpose, the MasterPure DNA purification kit (Epicentre, Madison, WI) was used. PCR assays targeting the rickettsial genes for the citrate synthase (*gltA*) and the outer membrane protein A (*ompA*), were performed (Table 1). For the citrate synthase gene (*gltA*), primer CS890r and a new primer (CS214\_for), designed by our team,

TABLE 1 Oligonucleotide primers

| PCR                      | Primer    | Nucleotide sequence (5'-3') | Position |
|--------------------------|-----------|-----------------------------|----------|
| <i>ompA</i>              | Rr190.70p | ATGGCGAATATTCTCCAAAA        | 70–90    |
|                          | Rr190.701 | GTTCCGTTAATGGCAGCATCT       | 701–681  |
| Real-time<br><i>ompA</i> | Rr190.314 | GGGCATTACTTACGGTGGTGAT      | 314–336  |
|                          | Rr190.630 | CTTTGACGGAGCTGCAGATTGTAT    | 630–607  |
| <i>gltA</i>              | CS214_for | ATCGAGGATATGATATTAAG        | 214–244  |
|                          | CS890r    | GCTTTAGCTACATATTTAGG        | 890–871  |

were used. For the *ompA* gene, a real-time PCR was designed by our team using a new pair of primers: Rr190.314 and Rr190.630. In addition, another pair of primers was used (Rr190.70 and Rr190.701). This set of primers amplifies a bigger fragment that includes the one amplified by Rr190.314 and Rr190.630. Real-time PCR assays were carried out and analyzed using a 7500 thermocycler (Applied Biosystems). PCR mixtures were set up in a UV-sterilized workstation. Measures to avoid contamination were carried out, including the use of separate and dedicated rooms for DNA extraction and molecular detection. All samples from each PCR were resolved by electrophoresis in 3% agarose gels in order to confirm the appropriate size. Amplification products were purified by ExoSAP-it (GE Healthcare, Buckinghamshire, United Kingdom). DNA obtained was sequenced using forward and reverse primers. DNA amplified by the primer pair Rr190.70p/Rr190.701 was also sequenced using Rr190.314 and Rr190.630. DNA se-

Received 23 February 2015 Returned for modification 31 March 2015

Accepted 14 June 2015

Accepted manuscript posted online 1 July 2015

Citation Nogueras MM, Roson B, Lario S, Sanfeliu I, Pons I, Anton E, Casanovas A, Segura F. 2015. Coinfection with “*Rickettsia sibirica* subsp. *mongolotimonae*” and *Rickettsia conorii* in a human patient: a challenge for molecular diagnosis tools. *J Clin Microbiol* 53:3057–3062. doi:10.1128/JCM.00457-15.

Editor: P. Bourbeau

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doi:10.1128/JCM.00457-15



quencing was performed on a 3130 genetic analyzer (Applied Biosystems) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Consensus sequences were established as the nucleotides that had been accurately read by at least two primers. Consensus sequences were compared with those in the GenBank DNA database by BLAST program. Phylogenetic analysis was performed using MEGA version 6.0 software (neighbor-joining method, Jukes-Cantor model). A test of phylogeny was performed using the bootstrap method (number of bootstrap replications, 1,000). As *gltA* is highly conserved among species which presented homology in BLAST, an outgroup was added to the tree (*Rickettsia felis* sequence corresponding to the same region) in order to show data in a more realistic way.

No amplification product was obtained directly from the blood sample. All PCRs yielded amplification products of the expected sizes both in biopsy specimen and in the liquid containing the biopsy specimen. Sequences obtained from the biopsy specimen were identical to those obtained from its liquid. Therefore, consensus sequences of *ompA* and *gltA* genes were built by SeqMan program considering all sequences from the biopsy specimen and the liquid. Nucleotide sequence analysis showed 100% identity with *ompA* gene of "*Rickettsia mongolotimonae*" HA-91 and *Rickettsia sibirica*. The sequence corresponding to *gltA* was 100% identical to sequences corresponding to *Rickettsia mongolotimonae*, *Rickettsia sibirica*, *Rickettsia* sp. strain BJ-90, *Rickettsia parkeri*, and Israeli tick typhus rickettsia. More detailed information is shown in Fig. 1 and 2. Identical sequences obtained from GenBank by the BLAST program were grouped together, as shown in Fig. 1 and 2. One FASTA sequence of each group together with sequences from the biopsy specimen and culture were analyzed using the MEGA program. Phylogenetic analysis for the *gltA* PCR target showed distance values of 0 among the biopsy specimen sequence and sequences from *Rickettsia mongolotimonae*, *Rickettsia sibirica*, *Rickettsia* sp., *Rickettsia parkeri*, and Israeli tick typhus rickettsia. As *gltA* is highly conserved among species of *Rickettsia*, a phylogenetic analysis for the *ompA* gene was also performed. This analysis grouped the sequence together with *R. mongolotimonae* DQ097082, strain HA-91 of *R. mongolotimonae* (U43796), and one strain of *R. sibirica* (HM050280) with a bootstrap value of 88. The other strains of *R. sibirica* were grouped separately.

Shell vials (SV) seeded with Vero cells were infected with the scalp eschar biopsy specimen and a heparin whole-blood sample. They were incubated with minimal essential medium (MEM) (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland) and 1 mM glutamine (Lonza,

Basel, Switzerland) in a 5% CO<sub>2</sub> at 32°C. On day 8, IFA was conducted using a serum containing antibodies against *R. conorii* and *R. slovaca*. Whereas SV inoculated with biopsy specimen were contaminated, two SV inoculated with blood were positive by IFA. The medium was transferred to a 25-cm<sup>2</sup> cultured flask. On day 16, rickettsial microorganisms were visualized by Gimenez staining. Cultures of *Rickettsia* spp. were established making serial subcultures. DNA was obtained from cultures and amplified using all the above-mentioned primer sets.

Consensus sequences of both *ompA* and *gltA* were 100% identical to those in different strains of *Rickettsia conorii*. Phylogenetic analysis for *gltA* PCR target showed distance values of 0 between the culture sample and *Rickettsia conorii*. As *gltA* is highly conserved among species of *Rickettsia*, a phylogenetic analysis for *ompA* was also performed. This analysis grouped the sequence together with *R. conorii* strains (bootstrap values of 77 to 98). In the phylogenetic analysis, the clade occupied by the isolate was distinct from that occupied by the biopsy specimen. Figures 1 and 2 show more detailed information.

This is an atypical clinical case of rickettsioses in a patient in whom two spotted fever group rickettsiae were detected. On the one hand, the clinical characteristics of the patient initially suggested a TIBOLA diagnosis. TIBOLA is caused mainly by *Rickettsia slovaca* and *Rickettsia raoultii* (1). Recently, other etiological agents related to this clinical entity have been described, and thus, the term SENLAT has been proposed (2, 3). Our case was similar to other clinical cases of TIBOLA from our area (4), where epidemiological studies have demonstrated the presence of *R. slovaca* (5–7). However, the patient did not have an epidemiological history that pointed to a *R. slovaca* infection. In addition, although some adults have been diagnosed, it had been observed that TIBOLA is predominantly a childhood disease (1, 4). On the other hand, MSF, endemic in our area, could be also possible, considering the contact with an infested dog and the exanthema. These symptoms are more typical of MSF and less frequent in TIBOLA (1, 8, 9). However, the exanthema is generally extended throughout the body in most MSF clinical cases, whereas it was observed only in the upper extremities of our patient. In addition, fever and high levels of transaminases could also suggest a MSF diagnosis (8, 9). The patient's condition improved after a regimen with doxycycline. This fact did not allow us to differentiate the etiological agent, as this treatment is useful in both infections (4, 10). Although serological results showed high titers of antibodies against

**FIG 1** Alignments and BLAST results corresponding to PCR assays targeting the gene for outer membrane protein A (*ompA*). Phylogenetic analysis was based on *ompA* sequencing of rickettsiae deposited in GenBank and detected in this study. \*, identical sequences have been grouped together. *R. mong.*, *R. mongolotimonae* (DQ097082); *R. sib-1*, *R. sibirica* (HM050280); HA-91, HA-91 (*R. sibirica* subsp. *mongolotimonae*) (U43796); RDL1, *Rickettsia* endosymbiont of *Amblyomma tuberculatum* isolate RDL1 (JF934878); *R. park-1*, *R. parkeri* (KJ158741, JQ906784); *R. afr-1*, *R. africae* (CP001612, EU622980, JN043509, U43790, U83436, GU247115); *R. afr-2*, *R. africae* (JQ691730); *R. afr-3*, *R. africae* (HQ335137, HQ335136, HQ335135, HQ335133, HQ335131); BJ-90, BJ-90 (*R. sibirica*) (AF179365); *R. slo-1*, *R. slovaca* (CP003375, CP002428, HM161786, HM161793, HM161788, HM161787, HM161797, HM161776, HM161769, HM161772, KF791234, KF791231, JQ691724, HM161770, KF791246, KF791241, KF791233, HM161798, KF791232, KF791236, KF791235, JQ691715, U43808, KF791242, KF791238, JQ691725); *R. afr-4*, *R. africae* (HQ335132); *R. sib-2*, *R. sibirica* (U43807); *R. park-2*, *R. parkeri* (KC003476, CP003341, KF782320, U43802); *R. afr-5*, *R. africae* (AB934397); *R. park-3*, *R. parkeri* (EU715288); *R. con-1*, *R. conorii* (U43791); *R. con-2*, *R. conorii* (KF245449, KF245450); *R. con-3*, *R. conorii* (KF245452, KF245451); Is. Tick, Israeli tick typhus rickettsia (AY197564, U43797, AY197566); *R. slo-2*, *R. slovaca* (JQ798909); *R. phil.*, *R. philipii* (CP003308); *R. slo-3*, *R. slovaca* (JQ798908); *R. afr-6*, *R. africae* (AB934396); *R. con-4*, *R. conorii* (AE006914); *R. con-5*, *R. conorii* (HM050291, DQ518245, U43806); *R. con-6*, *R. conorii* (KF245453); *R. con-7*, *R. conorii* (U43794); *R. con-8*, *R. conorii* (U01028); *R. rick.*, *R. rickettsii* (CP003318, CP003309, CP003307, CP003306, CP003305, CP000848, M31227). \*\*, percent identity of sequences obtained when homologies were searched with the MEGABLAST program. NB, no BLAST (sequences that did not appear when a MEGABLAST search was performed). Bootstrap values of >50 are shown (1,000 bootstrap replications).

[illegible]

**FIG 2** Alignments and BLAST results corresponding to PCR assays targeting the gene for citrate synthase (*glta*). Phylogenetic analysis was based on *glta* sequencing of rickettsiae deposited in GenBank and detected in this study. \*, identical sequences were grouped together. R. sib-1, *R. sibirica* (KM289711, JX945526, U59734); R. park, *R. parkeri* (KF782319, KM245157, KJ158742, JQ906783, CP003341, JN126320, EF102236, U59732); R. mong, *R. mongolotominae* (DQ097081), Is. Tick, Israeli tick typhus *Rickettsia* (U59727); R.slo, *R. slovacica* (KJ410267, CP003375, CP002428, U59725); R.af-1, *R. africana* (JN043505); R. sib-2, *R. sibirica* (HM050279, HM050296); R. con-1, *R. conorii* (HM050292, AE006914, EU716648, U59730); R. marm, *R. marmionii* (AY737684); R. con-2, *R. conorii* (U59728); R. honei (U59726, AF018074); R. peak, *R. peacockii* (HF935076, CP001227, DQ100162); R. afr-2, *R. africana* (HQ335125); R. afr-3, *R. africana* (HM050288, CP001612); HA-91, HA-91 (*R. sibirica* subsp. *mongolotominae*) (U59737); R. rick, *R. rickettsii* (KJ735644, KF472602, JN393861, JN393860, JN393859, JN393858, JN393857, JN393856, JN393855, JN393854, CP003318, CP003311, CP003309, CP003307, CP003306, CP003305, CP000766, CP000848, U59729, DQ402514, DQ150686, DQ150683); R. rao-1, *R. raoultii* (JX835455, DQ365804); R. phil, *R. philipii* (CP003308); R. jap, *R. japonica* (AP011533); R. heil, *R. heilongjiangensis* (CP002912, AY280709, AB473994, AB473993, AB473992, AB473812, AB516964, AY285776); R. rao-2, *R. raoultii* (KM288494, KM288493, KM288492, KM288491, KM288490). \*\*, percent identity of sequences obtained when homologies were searched with the MEGABLAST program. NB, no BLAST (sequences that did not appear when MEGABLAST was performed). Bootstrap values of >50 are shown (1,000 bootstrap replications).

*R. conorii* and negative results concerning *R. slovaca*, data were again not conclusive, for two main reasons: a long period is required for seroconversion in *R. slovaca* infections, and members of the genus *Rickettsia* can exhibit cross-reactivity (1).

The isolation of *R. conorii* from the blood sample was evidence that the patient's disease was MSF. On the other hand, "*Rickettsia sibirica* subsp. *mongolotimonae*" was detected in the biopsy specimen by molecular techniques. *R. sibirica* subsp. *mongolotimonae*

was described as a human pathogen in 1996 (11). Since then, other cases have been described in different countries (12–15), including Spain (16–18). Although it is mainly associated with *Hyalomma* spp. (1), it has been detected in *Rhipicephalus* spp. (13, 15, 18), which could be an emerging vector for this microorganism. *R. sibirica* subsp. *mongolotimonae* is the etiological agent of lymphangitis-associated rickettsiosis (LAR). Clinical cases usually occur in the spring and summer, and it has a wide variety of symptoms. Most typical symptoms are fever, myalgia, rash, headache, enlarged lymph nodes, and lymphangitis, although none of them are present in 100% of patients. Severity of disease ranges from mild symptoms to some complications, although no fatality has been reported so far (1). The presence of enlarged lymph nodes, face swelling, and the seasonality of our case were compatible with a *R. sibirica* subsp. *mongolotimonae* infection, despite the lack of lymphangitis. In fact, this case had some features similar to those described recently in another Mediterranean coast region of Spain (17). Recently, *R. sibirica* subsp. *mongolotimonae* was described as another etiological agent of SENLAT (3); thus, it is not surprising that some clinical features of our case initially suggested a TIBOLA/SENLAT diagnosis. Moreover, even though most SENLAT patients described were young, a second group of patients whose ages range from 40 to 70, consistent with our patient's age, has been described (2).

It has been demonstrated that more than one microorganism can coexist in ticks (19). The fact that two members of spotted fever group rickettsiae were found in the same patient should not be surprising, considering that they could share the same vector (13, 15, 18). Both microorganisms found in our patient are present in our country (8, 9, 16–18). In addition, *R. sibirica* subsp. *mongolotimonae* has been detected in *Rhipicephalus* spp., the main vector of *R. conorii* (13, 15, 18). In fact, in Spain, *R. sibirica* subsp. *mongolotimonae* was found in 3.7% of *Rhipicephalus pusillus* and in 3.6% of *Rhipicephalus bursa* ticks, whereas no *Hyalomma* spp. contained this microorganism (18). Unfortunately, the patient did not carry any tick at admission. Therefore, the actual vector of this clinical case could not be determined.

There is scarce information on coinfections in humans. However, some cases of human tick-borne coinfections have been described (19). Dual infections with *Rickettsia* spp. and members of other genera have been described (10, 20), as well as coinfection among *Rickettsia* species (21, 22).

This case shows that not only should clinicians consider dual infections in patients with an atypical diagnosis of tick-borne disease, but also more specific techniques for diagnosis, such as culture or molecular detection, are useful tools to determine the etiology of rickettsioses. New molecular techniques, as well as the improvement of cell culturing, will lead us to new insights into the clinical and epidemiological characteristics of rickettsial diseases and will help us to detect mixed infections, which are likely much more common than has been thought.

**Nucleotide sequence accession numbers.** Consensus sequences were deposited in GenBank under accession numbers [KT345980](#) (*ompA* biopsy specimen), [KT345978](#) (*ompA* culture from blood), [KT345979](#) (*gltA* biopsy specimen), and [KT345977](#) (*gltA* culture from blood).

## ACKNOWLEDGMENTS

We thank David Miñana for his support with the phylogenetic analysis.

We have no conflicts of interests to declare.

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